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^{2}H-NMR, ^{31}P-NMR and DSC characterization of a novel lipid organization in calcium-dioleoylphosphatidate membranes. Implications for the mechanism of the phosphatidate calcium transmembrane shuttle

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^{2}H-NMR, ^{31}P-NMR and DSC investigations are presented on the structure and dynamics of the Ca^{2+}-dioleoylphosphatidate complex which is formed upon addition of calcium to dispersions of pure dioleoylphosphatidate or of dioleoylphosphatidate in mixtures with dioleoylphosphatidylcholine (DOPC). It is concluded that the phosphate region in the polar headgroup of dioleoylphosphatidate is immobilized, while the oleate chains remain liquid and have increased disorder. In mixtures of dioleoylphosphatidate and DOPC in the presence of calcium a dioleoylphosphatidate-rich phase is segregated, in which the molecular behaviour of phosphatidate is rather similar to that of the pure Ca^{2+}-dioleoylphosphatidate complex. A hypothetical model is proposed for the structure of this complex and this is correlated with the dioleoylphosphatidate-mediated transmembrane transport of calcium (Smaal, E.B., Mandersloot, J.G., De Kruijff, B. and De Gier, J. (1986) Biochim. Biophys. Acta 860, 99–108). Data indicate that this transmembrane shuttle is an inverted organization of phosphatidate molecules enclosing calcium ions in an anhydrous core.

Introduction

Addition of calcium to model membranes containing the negatively charged phospholipid phosphatidic acid results in a number of remarkable changes in the structure and properties of the membrane [1–11]. In two earlier studies [1,2] we reported on the permeability behaviour [1], and on the calcium-membrane and membrane-membrane interaction [2] in the presence of calcium for dioleoylphosphatidylcholine (DOPC) vesicles containing 20 mol% of dioleoylphosphatidate. Summarizing, we concluded that the selective permeability increase for calcium and calcium chelators

Abbreviations: PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; DEPA, dielaidoylphosphatidate; DMPA, dimyristoyl PA; DPPA, dipalmitoyl PA; DOPC, dioleoyl PC; DOPG, dioleoyl PG; POPG, palmitoyloleoyl PG; DOPS, dioleoyl PS; MLV, multilamellar vesicles; T_c, phase transition temperature; $\Delta\nu_q$, quadrupolar splitting; Pipes, 1,4-piperazinediethanesulfonic acid.

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observed at low calcium concentrations (< 2.5 mM) [1] is a direct consequence of calcium–phosphatidate interactions, but not of intermembrane contacts [2]. For the mechanism of the translocation of calcium and its chelators it was proposed that dehydrated uncharged complexes of calcium, phosphatidate and, possibly, calcium chelator are acting as transmembrane shuttles [2–4]. The observed increased permeability for calcium appeared to be selective for phosphatidate, because when phosphatidylglycerol was used instead no permeability changes were observed upon calcium addition [1].

The aim of the present study is to provide a structural basis for the permeability data by elucidation of the behaviour of dioleoylphosphatidate in a complex with calcium at the molecular level. The calcium concentration dependency of the thermotropic phase behaviour of dioleoylphosphatidate and DOPC in pure and in mixed systems was investigated by differential scanning calorimetry. Further characterization of the physical state and organization of the hydrophobic part of dioleoylphosphatidate (or DOPC) was carried out by ^{2}H-NMR, using phospholipids dideuterated at the 11-position of both oleate chains. The local order in the phosphate region of dioleoylphosphatidate (and DOPC) was characterized using ^{31}P-NMR techniques. To show the specificity of the Ca^{2+}–dioleoylphosphatidate interactions the results are compared with those of DOPG.

It is shown that at pH 7.4 in the presence of calcium dioleoylphosphatidate, in pure and also in mixed systems with DOPC, adopts an organization which is unique among the known phospholipid molecular structures. A structure is envisaged in which calcium forms a dehydrated complex with the polar headgroup of dioleoylphosphatidate resulting in the immobilization of the headgroup region, whilst the disorder of the acyl chain region is increased.

Materials and Methods

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) (sodium salt) and 1,2-dioleoyl-*sn*-glycero-3-phosphate (dioleoylphosphatidate) (sodium salt) were synthesized and purified as described earlier [1,12]. 1,2-[11,11-$^2H_2$]DOPC was synthesized as described by Chupin et al. [13] and 1,2-[11,11-$^2H_2$]dioleoylphosphatidate (sodium salt) was synthesized from 1,2-[11,11-$^2H_2$]DOPC [1]. All phospholipids were chemically pure (> 99%) as indicated by HPTLC and contained no detectable calcium (< 0.5 mol%) as measured by atomic absorption spectrophotometry.

The calcium salt of L-glycerophosphate was prepared by mixing an aqueous solution of the disodium salt (at pH 7.4) and a 1 M $CaCl_2$ solution. The Ca^{2+}-glycerophosphate which precipitated was filtered, washed and dried.

Differential scanning calorimetry

The thermotropic phase behaviour of phospholipids in aqueous dispersion was determined by DSC (Perkin-Elmer, DSC-4). Multilamellar vesicles (MLV; 10 μmol of lipid phosphorus [14]) were prepared in 1.5 ml of a solution containing 100 mM NaCl, 25 mM Pipes (pH 7.4), 40 μM EDTA, 7 μg/ml of the calcium ionophore A23187 (excess) and 0–75 mM $CaCl_2$. In some experiments the buffer was mixed with 50 vol% ethylene glycol. After 30 min of incubation at 25°C, the MLV were pelleted by centrifugation (20 min; 40 000 × *g*), the pH of the supernatant was checked and the wet pellets were transferred into stainless steel sample pans (volume about 75 μl). The thermograms were obtained using a scanning rate of 2 Cdeg/min.

Nuclear magnetic resonance (NMR)

MLV (50–100 μmol of lipid phosphorus [15]) were prepared in 10 ml of a 100 mM NaCl, 25 mM Pipes, 40 μM EDTA solution (pH 7.4). These were pelleted by centrifugation (20 min; 40 000 × *g*) and the pH of the supernatant was checked. After resuspending the pellets to a final volume of 0.7–1.0 ml, 30 μg A23187 was added (as a 1 mg/ml solution in methanol), and the suspension was transferred into 10 mm (o.d.) NMR tubes, except for the ^{31}P cross polarization experiments, which were performed using 7.5 mm (o.d.) tubes. When needed, calcium was added from a 100 mM or 1 M $CaCl_2$ (pH 7.4) stock solution. Unless otherwise indicated, the NMR experiments were carried out at 25°C.

^{2}H-NMR. ^{2}H-NMR spectra were recorded at 46.1 MHz on a Bruker MSL 300 spectrometer by employing the quadrupolar echo sequence [15]. The 90° pulse was 13 μs, the 90° pulse separation was 50 μs, acquisition of the echo was started 57 μs after the the second 90° pulse while a dwell time of 8 μs was used. Recycle delays were 0.2 s and 2500–7500 echos were accumulated for each spectrum. In some cases a solenoidal high-power probe (90° pulse width is 2.3 μs) was used.

^{31}P-NMR. ^{31}P-NMR spectra were recorded at 121.49 MHz on the above spectrometer. Unless otherwise indicated, a single-pulse experiment was used in a high-resolution probe with 90° pulse of 13 μs and a 1 s recycle time. High-power proton decoupling was employed during acquisition. For the dry powder of Ca^{2+}-glycerophosphate and the wet Ca^{2+}-dioleoylphosphatidate complex the cross-polarization technique [16] was used in a 7.5 mm solenoidal probe for sensitivity enhancement. A 5 μs 90° proton pulse was followed by a 1 ms contact time during which ^{1}H and ^{31}P radiofrequency fields were present at levels to fulfill the Hartmann-Hahn condition. A 4 s recycle delay was used.

If necessary, total intensities of ^{2}H- and ^{31}P-NMR spectra were determined by computer integration. In order to allow direct comparison of the intensities measured from subsequent spectra in a Ca^{2+}-titration series, it was assured that the total sample volume was considerably smaller than the volume enclosed by the radiofrequency coil.

Results

DSC of dioleoylphosphatidate, dioleoylphosphatidylcholine and dioleoylphosphatidylglycerol dispersions in the absence and presence of calcium

Figs. 1 and 2 show the thermotropic phase behaviour of DOPC, dioleoylphosphatidate and DOPG. The gel to liquid-crystalline phase transition for DOPC and dioleoylphosphatidate was found at -20°C and −8°C, respectively, with corresponding enthalpies (ΔH) of 8.5 kcal/mol and 5.5 kcal/mol (Fig. 1, left panel). The higher T_c and lower ΔH of dioleoylphosphatidate compared to those of DOPC may be explained by a difference in molecular packing of phosphatidate as a consequence of its relatively small polar region and the presence of intermolecular hydrogen bonds [3,18–21]. To improve the resolution of the phase transition peak of dioleoylphosphatidate the ice-water transition was suppressed by the addition of 50 vol% ethylene glycol (Fig. 1, right panel). In the presence of the anti-freeze the transition temperature (T_c) of DOPC is shifted upward (to −14°C; ΔH 13.3 kcal/mol) and that of dioleoylphosphatidate seems to be shifted downward (to −11°C; ΔH 6.6 kcal/mol). Among other things, these shifts in the T_c may be caused by an influence of ethylene glycol on the acyl chain packing, as was observed with ^{2}H-NMR [17].

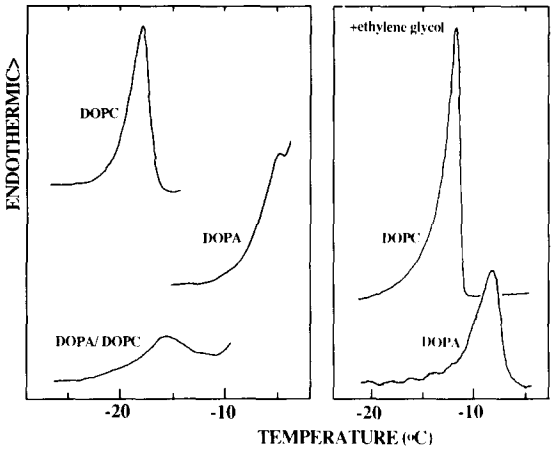

Fig. 1. Thermograms of dispersions of DOPC and dioleoylphosphatidate (DOPA), pure and in an equimolar mixture in the absence and the presence of 50 vol% ethylene glycol. Depicted are the DSC scans of dioleoylphosphatidate/DOPC (50:50, mol%) MLV (left panel) and those of pure dioleoylphosphatidate and DOPC vesicles prepared in the absence (left panel) and the presence (right panel) of 50 vol% of ethylene glycol. All scans are presented on the same scale, except the one of dioleoylphosphatidate in the absence of ethylene glycol (left panel), for which a 4-times-lower sensitivity was used. Experimental details are described in Materials and methods.

For DOPG a more complex thermotropic phase behaviour was found (Fig. 2). In a DSC cooling scan of a dispersion of the sodium salt of DOPG from 40°C to −35°C an exothermic phase transition was found at −21.5°C (ΔH 9.7 kcal/mol; Fig. 2, curve a), which is roughly comparable to the transition temperature of DOPC. Subsequent heating (Fig. 2, curve b) resulted in an initial tendency for an endothermic phase transition at the same temperature (−21.5°C), passing into an exothermic transition (−18.3°C) with a rather

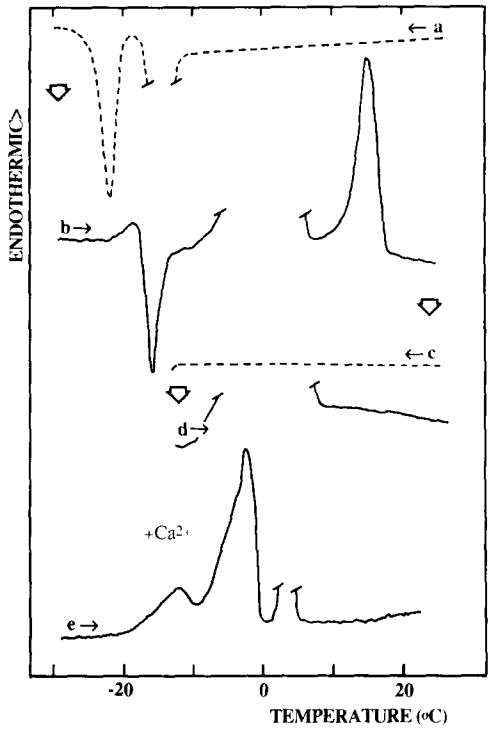

Fig. 2. Thermotropic phase behaviour of DOPG in the presence and absence of $CaCl_2$. In the absence of calcium the following thermograms were recorded: curve a, cooling scan (40°C → −35°C); curve b, subsequent heating scan (−35°C → 25°C); curve c, subsequent cooling scan (25°C → −18°C); and (after isothermical freezing of the aqueous solution) curve d, subsequent heating scan (−18°C → 25°C). Furthermore, curve e is the heating scan of DOPG prepared in the presence of calcium (80 mM of free Ca^{2+}). Experimental details are described in Materials and Methods.

high enthalpy (ΔH approx. 8 kcal/mol). After further scanning to higher temperatures a second endothermic transition was found at 11.3°C (ΔH 14.8 kcal/mol). No lipid phase transitions were found scanning from 40°C to −18°C and (after isothermic freezing of the aqueous solution) back (Fig. 2, curve c and d) or from −5°C to −35°C (after scanning from −35°C to −5°C; results not shown). DSC of DOPG in the presence of 50 vol% ethylene glycol showed a similar complex behaviour with somewhat higher phase transition temperatures, but did not reveal any additional phase transitions (results not shown).

The unusual thermotropic phase behaviour of DOPG is probably due to the occurrence of a metastable gel phase which is formed after cooling of the sample below −21.5°C. This metastable phase is converted into a more stable gel phase with a more efficient acyl chain packing after subsequent heating to above −18°C. Further heating results in a transition into a liquid-crystalline phase (at 11.3°C).

We next investigated the effect of calcium on the thermotropic behaviour of DOPC, dioleoylphosphatidate and DOPG. Upon addition of calcium up to 35 mM the DSC characteristics of DOPC do not change significantly (data not shown). In contrast with the finding for the sodium salt of DOPG, the DSC behaviour of the calcium salt of DOPG showed no hysteresis (Fig. 2, curve e; cooling scan not shown). The heating scan of DOPG in the presence of calcium shows two phase transition peaks (−17°C and −6°C; ΔH 4 and 18 kcal/mol, respectively) (Fig. 2, curve e). The origin of these two peaks is not clear. Addition of 50 vol% ethylene glycol to the sample did not reveal an additional phase transition in the temperature range of −35°C to 100°C (results not shown).

Addition of calcium to dioleoylphosphatidate dispersion causes an abolishment of the gel to liquid-crystalline phase transition in the temperature range from −35°C to 100°C, both in the absence and in the presence of 50 vol% ethylene glycol (data not shown). From these findings it is not clear whether the calcium salt of dioleoylphosphatidate is in the gel phase at room temperature as has been found for the calcium salt of the disaturated species of phosphatidic acid [11,18,22,23], PG [18,24], egg PC-derived phosphatidic acid [25] and various species of PS [18,26,27], or in the liquid-crystalline phase, as has been observed for the calcium salt of bovine heart cardiolipin [28].

From these data it can be concluded that at 25°C, at which temperature dioleoylphosphatidate shows its calcium translocating properties, in the absence of calcium DOPC, DOPG and dioleoylphosphatidate all are in a liquid-crystalline state. Upon addition of calcium at 25°C DOPC and DOPG remain fluid, while the physical state of dioleoylphosphatidate can not be derived from the DSC data. To reach further understanding in this respect ^{2}H-NMR studies were done on dioleoylphosphatidate deuterated in the acyl chains.

^{2}H-NMR of pure dioleoylphosphatidate dispersions

In Fig. 3 the ^{2}H-NMR spectra are shown of hydrated 1,2-[11,11-$^2H_2$]dioleoylphosphatidate in the absence and presence of increasing amounts of calcium at pH 7.4. The depicted free calcium concentrations are calculated from the total amount of added calcium and the phospholipid concentration, using the calcium binding curve as presented in Ref. 2 (K_d 1.7 mM, maximal stoichiometry 0.6 nmol Ca^{2+}/nmol phosphatidate). In the absence of calcium the ^{2}H-NMR spectrum of dioleoylphosphatidate is an axially symmetric powder pattern which has a residual quadrupolar splitting ($\Delta\nu_q$) of 5.1 kHz. The sharp peak at a frequency of 0 kHz represents deuterons present at natural abundance in the aqueous solution. At increasing calcium concentrations, the ^{2}H-NMR spectra show a gradual loss in intensity of the original doublet, while a peak with a broad isotropic line shape increases in intensity (Fig. 3). At calcium concentrations of 4.5 mM and higher the original doublet signal is completely lost, and only the broad isotropic signal remains. It is important to note that the total integrated ^{2}H-NMR signal intensity decreases only slightly upon calcium addition, such that at 4.5 mM of calcium 88% of the original intensity is left (Fig. 3; see also Fig. 5). In order to assure that the experimental set-up (90°C pulse of 13 μs) employed, enabled homogeneous acquisition of the entire signal, we have also measured the latter sample (i.e., at 4.5 mM of calcium) in a high-power solenoidal probe (90° pulse of 2.3 μs). The intensity distribution was identical in both cases, demonstrating that there are no broad components escaping detection in the set-up routinely used.

The temperature dependencies of the ^{2}H-NMR spectra of the sodium and calcium salt of 1,2-[11,11-$^2H_2$]dioleoylphosphatidate are shown in Fig. 4. To prevent freezing of the samples 50 vol% ethylene glycol was added. The presence of the antifreeze causes a decrease in $\Delta\nu_q$ [17]. For the

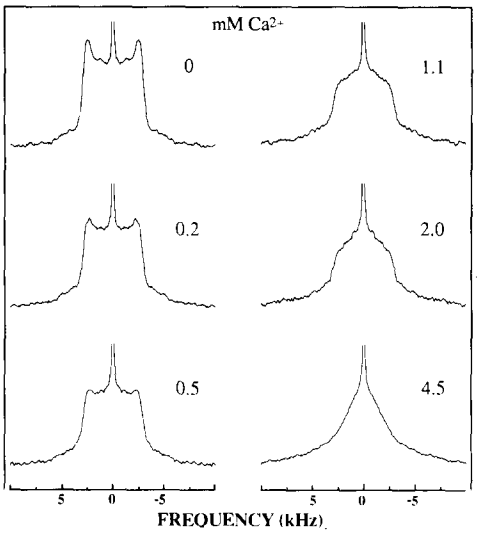

Fig. 3. 46.1 MHz ^{2}H-NMR spectra of 1,2-[11,11-$^2H_2$]dioleoylphosphatidate at various calcium concentrations. The sharp peak at 0 kHz in all spectra is caused by deuterons present at natural abundance in the aqueous solution. For experimental details see Materials and Methods.

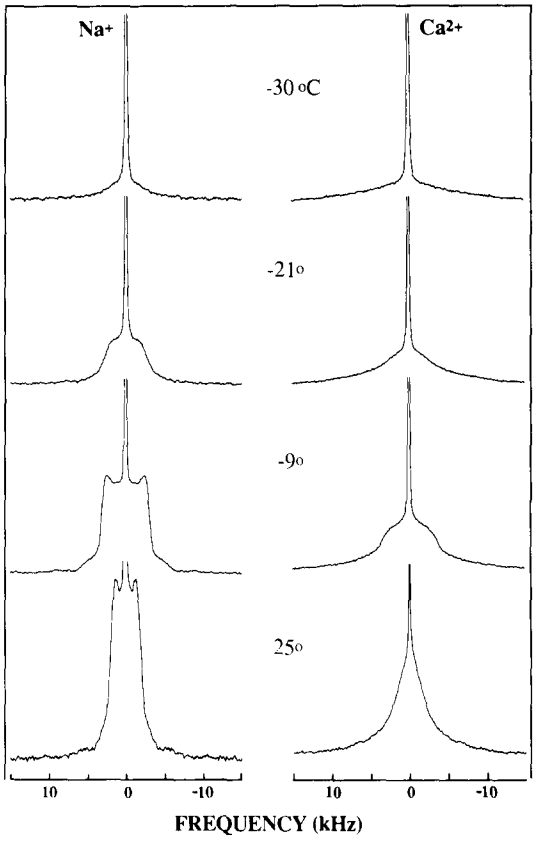

Fig. 4. Temperature-dependency of the 46.1 MHz ^{2}H-NMR spectra of the sodium salt (left column) and the calcium salt (right column) of 1,2-[11,11-$^2H_2$]dioleoylphosphatidate. The spectra were recorded after 45 min of equilibration at the desired temperature. The experimental conditions are described in Materials and Methods.

sodium salt of the deuterated dioleoylphosphatidate lowering of temperature from 25°C to −9°C results in an increase in $\Delta\nu_q$ from 2.8 kHz to 5.3 kHz, while the line shape and the signal intensity do not change significantly (Fig. 4). In the temperature range in which according to the DSC results (Fig. 1, right panel) the gel to liquid-crystalline phase transition temperature is passed, a dramatic change in shape and loss of intensity of the ^{2}H-NMR signal is observed (Figs. 4 and 5). For pure 1,2-[11,11-$^2H_2$]DOPC a similar temperature dependency of the line shape (results not shown) and intensity (Fig. 5) of the ^{2}H-NMR signal was found. However, the signal of the calcium salt of 1,2-[11,11-$^2H_2$]dioleoylphosphatidate shows a gradual broadening with decreasing temperature in the range of −10°C to −30°C (Fig. 4), lacking the drop in signal intensity at about −15°C which was observed for the sodium salt (Fig. 5).

^{31}P-NMR of pure dioleoylphosphatidate dispersions

For a further characterization of the interaction of calcium with phosphatidate ^{31}P-NMR techniques were used to probe changes in dynamics in the phosphate region. Fig. 6A shows the ^{31}P-NMR spectrum of the sodium salt of dioleoylphosphatidate at pH 7.4. The spectrum has an asymmetric line shape with a low-field shoulder and a high-field peak separated by approximately 35 ppm, corresponding to an axially symmetric chemical shift anisotropy tensor. Upon calcium introduction to a free concentration of 4.5 mM, the ^{31}P signal is greatly reduced in intensity (Fig. 4B): 30% of the original intensity is left under these experimental conditions. This reduction might be due to immobilization of the phosphate in dioleoylphosphatidate [29]. To get more information about the nature of the physical state of the Ca^{2+}-dioleoylphosphatidate system, we have employed the ^{31}P-NMR cross-polarization technique

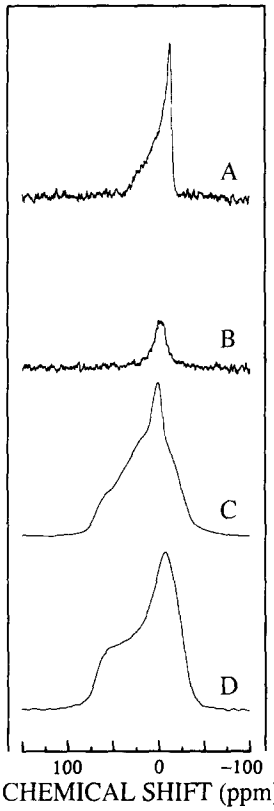

Fig. 6. 121.49 MHz ^{31}P-NMR spectra of dispersions of dioleoylphosphatidate (Na^+), dioleoylphosphatidate (Ca^{2+}) and the dry powder of calcium glycerophosphate. Shown are single-pulse ^{31}P-NMR spectra of the sodium salt (A) and the calcium salt (free [Ca^{2+}] = 4.5 mM) (B) of dioleoylphosphatidate, and cross polarization ^{31}P-NMR spectra of the calcium salts of (wet) dioleoylphosphatidate (C) and (dry) glycerophosphate (D), respectively. Spectra A and B are depicted on the same vertical scale. For experimental details see Materials and Methods.

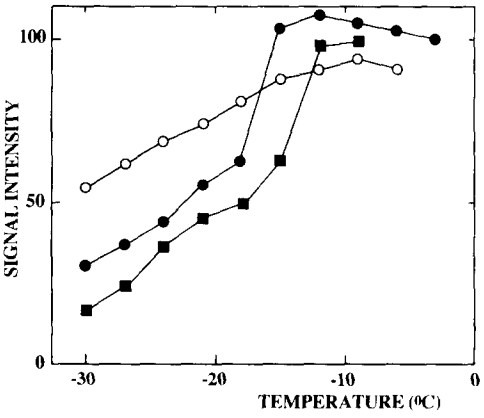

Fig. 5. Temperature-dependency of the signal intensity of the 46.1 MHz ^{2}H-NMR spectra of the sodium salt (●) and calcium salt (○) of 1,2-[11,11-$^2H_2$]dioleoylphosphatidate and 1,2-[11,11-$^2H_2$]DOPC (■). The results are expressed in arbitrary units. The data of dioleoylphosphatidate (Ca^{2+}) are normalized to those of dioleoylphosphatidate (Na^+) and are therefore directly comparable. For details see the legends of Fig. 4 and Materials and Methods.

[16] which efficiently enhances the sensitivity for ^{31}P nuclei in an immobilized configuration. For the dioleoylphosphatidate calcium salt a broad rigid lattice lineshape, spanning approx. 80 ppm, is observed through this approach (Fig. 4C). For comparison, Fig. 4D shows the ^{31}P cross-polarization spectrum of a dry powder of the calcium salt of L-glycerophosphate. This spectrum corresponds to a chemical shift anisotropy tensor similar to that of the calcium salt of dioleoylphosphatidate in aqueous buffer, while also both spectra deviate slightly from axial symmetry. In contrast, the ^{31}P cross-polarization spectrum from a dry powder of the sodium salt of L-glycerophosphate is fully axially symmetric (data not shown). It should be noted that the ^{31}P-NMR spectrum of DOPG (with a chemical shift anisotropy of 35 ppm) which corresponds with a liquid-crystalline bilayer organization of the lipid, does not change upon addition of calcium (data not shown).

The dioleoylphosphatidate / dioleoylphosphatidylcholine mixed systems in the absence and presence of calcium

In Fig. 7 the results are depicted of DSC, ^{2}H-NMR and ^{31}P-NMR studies on the equimolar mixed systems of dioleoylphosphatidate and DOPC. To make the findings of the different techniques used mutually comparable, the results are presented on the basis of the calculated free calcium concentrations. The gel to liquid-crystalline phase transition (DSC) of the system in the absence of calcium shows a rather broad peak at temperatures in between the transition temperature of both lipids in the pure form (Fig. 7, 1st column). Somewhat surprising is that the transition enthalpy of the mixture (4.1 kcal/mol) is much lower than the theoretical average of the enthalpies of pure dioleoylphosphatidate and DOPC (5.5 kcal/mol and 8.5 kcal/mol, respectively). In the thermotropic behaviour of the system with increasing calcium concentration three stages can be recognized. At low millimolar calcium concentrations a shift of the phase transition to lower temperatures is observed, combined with a decrease in transition enthalpy to about 3.0 kcal/mol, which may suggest a withdrawal of a fraction of the molecules from the phase transition. At further increasing calcium concentration the broad transition is splitting up in a sharp peak ($T_c = -19°C$), which is appearing at about the same temperature as the transition of pure DOPC, and a minor shoulder at lower temperature. This strongly suggests a phase separation leading to a DOPC-rich phase. At 31 mM of free calcium the phase transition at the low temperature side is somewhat increased in enthalpy at the cost of the higher temperature transition, whilst the total enthalpy is further decreased to 1.3 kcal/mol.

The ^{2}H-NMR spectra of the dioleoylphosphatidate/DOPC (50:50, mol%) dispersion in which only the phosphatidate is deuterated (Fig. 7, 2nd column) show a similar gradual change in line shape upon calcium addition as is found for the pure 1,2-[11,11-$^{2}H_2$]dioleoylphosphatidate, be it at higher calcium concentrations. In the absence of calcium the $\Delta\nu_q$ measured from the spectrum of dioleoylphosphatidate in the mixture (5.5 kHz) is somewhat higher than that of the pure system (5.1 kHz). At increasing calcium concentration the doublet component of the original spectrum disappears, while a more isotropic component is emerging. This is accompanied by a small loss of total echo intensity (15% at 22 mM of free Ca^{2+}).

The ^{2}H-NMR spectrum of the equimolar mixture in which DOPC is exclusively deuterated displays a $\Delta\nu_q$ of 6.3 kHz in the absence of calcium (Fig. 7, 3rd column). This is higher than the $\Delta\nu_q$ of the pure deuterated DOPC (5.5 kHz). The calcium concentration dependency of the spectrum of dispersions of 1,2-[11,11-$^{2}H_2$]DOPC and dioleoylphosphatidate in an equimolar mixture shows similarities with the results for deuterated dioleoylphosphatidate, albeit much higher calcium concentrations are needed to reach the same effect (Fig. 7, 3rd column).

The ^{31}P-NMR spectrum of dioleoylphosphatidate/DOPC (50:50, mol%) dispersions shows a lineshape which is corresponding to an axially symmetric chemical shift anisotropy tensor, characteristic of phospholipids organized in extended bilayers. At 22 mM of calcium a small isotropic signal is superimposed on the bilayer line shape, while the total signal intensity is decreased with about 35% (Fig. 7, 4th column).

The thermotropic behaviour of dioleoylphosphatidate/DOPC (20:80, mol%) dispersion as a function of the calcium concentration is shown in

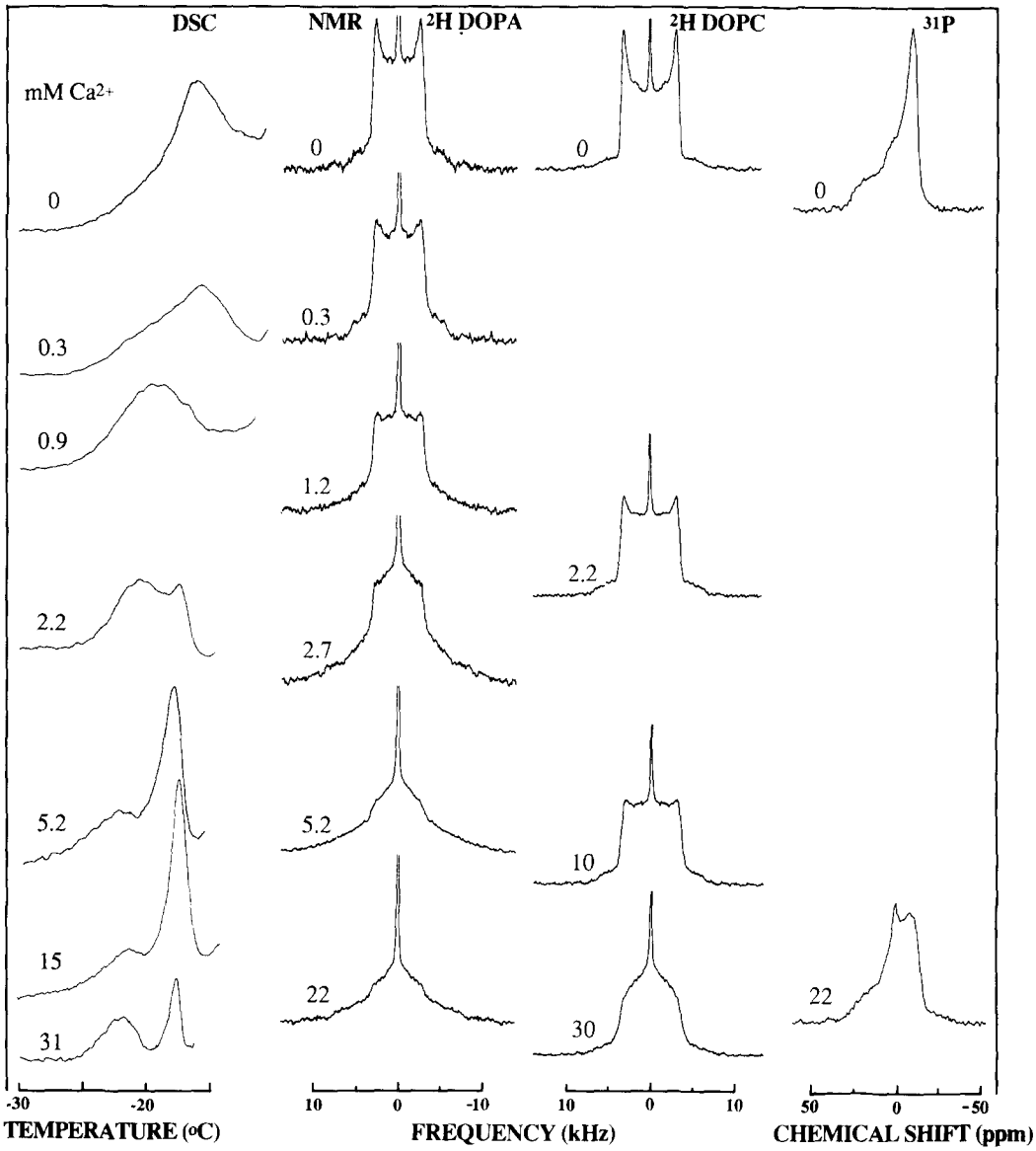

Fig. 7. Structural phase behaviour of dioleoylphosphatidate (DOPA) and DOPC in an equimolar mixture as a function of the calcium concentration. Depicted are the thermotropic phase behaviour (DSC heating scans) (1st column), the 46.1 MHz ^{2}H-NMR spectra of 1,2-[11,11-$^2H_2$]dioleoylphosphatidate/DOPC (2nd column) and dioleoylphosphatidate/1,2-[11,11-$^2H_2$]DOPC (3rd column), and the single-pulse 121.49 MHz ^{3}P-NMR spectra (4th column) of dioleoylphosphatidate/DOPC (50:50, mol%) dispersions. The free calcium concentrations are indicated on the left side of each curve. The experimental conditions are described in Materials and Methods.

Fig. 8. Here also a minor shift to lower temperatures of the phase transition is observed with increasing calcium concentrations. Furthermore, the transition enthalpy at higher calcium concentrations is about 25% lower than the enthalpy found without calcium. ^{2}H-NMR measurements in the absence of calcium of the mixture in which the phosphatidate is labelled show an axially symmetric powder pattern with a $\Delta\nu_q$ of 5.9 kHz (Fig. 9A). The presence of calcium causes again an increasing intensity in the middle part of the spectrum, while the total echo intensity shows a small

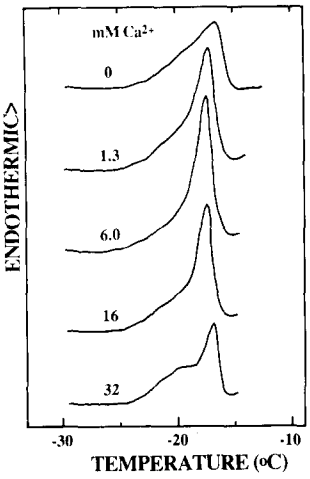

Fig. 8. Thermograms of dispersions of dioleoylphosphatidate/DOPC (20:80, mol%) at various calcium concentrations. For experimental conditions see Materials and Methods.

decrease of 11%. However, the effect of calcium on the dioleoylphosphatidate lineshape for this system is smaller than for the equimolar mixture of dioleoylphosphatidate and DOPC. The ^{31}P-NMR spectra of the dioleoylphosphatidate/DOPC (20:80, mol%) dispersions are identical in the absence and presence of calcium (Fig. 9C and D, respectively), while there is no significant loss in the total integrated signal intensity.

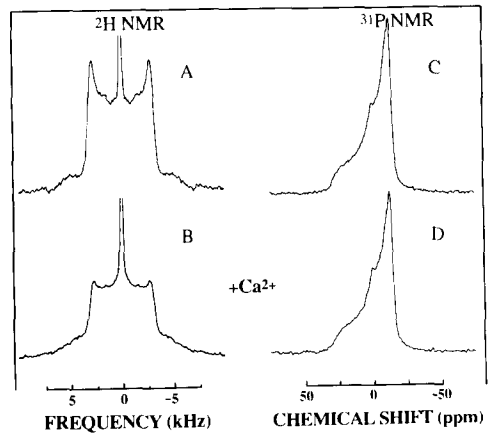

Fig. 9. 46.1 MHz ^{2}H-NMR (A,B) and 121.49 MHz ^{31}P-NMR (C,D) spectra of dispersions of 1,2-[11,11-$^{2}H_2$]dioleoylphosphatidate/DOPC (20:80, mol%) in the absence (A,C) and the presence (B,D) of 10 mM of free calcium. For experimental conditions see Materials and Methods.

Discussion

The aim of this study is the elucidation of the molecular structure of the calcium-phosphatidate complex, which is thought to act as a transmembrane calcium shuttle [1–3,19]. We are especially interested in the properties of the complex under conditions comparable to those we used in earlier studies (dioleoylphosphatidate/DOPC (20 : 80, mol%) system, at pH 7.4). However, firstly we will discuss earlier findings on the macroscopic phase behaviour of pure Ca^{2+}-dioleoylphosphatidate, followed by an interpretation of our results on the structural behaviour of pure dioleoylphosphatidate in the presence of calcium. Subsequently, the characterization of the dioleoylphosphatidate/DOPC mixed system will be discussed, resulting in a preliminary model of the phosphatidate calcium transmembrane shuttle.

Macroscopic phase properties of dioleoylphosphatidate in the absence and presence of calcium

Our ^{2}H-NMR and ^{31}P-NMR results and previous freeze-fracture electron microscopy results [8,9] indicate that in the absence of calcium at pH 7.4 phosphatidate organizes in lamellar structures. From earlier studies on the structural phase behaviour of dioleoylphosphatidate upon addition of calcium, using freeze fracture electron microscopy [8,9], ^{2}H-NMR [9], ^{31}P-NMR [9,30] and X-ray scattering [30], it was concluded that the organization of Ca^{2+}-dioleoylphosphatidate is highly dependent on pH. Farren et al. [9] found that at pH 5.5 in the presence of calcium dioleoylphosphatidate adopts a hexagonal H_{II} organization [9], which is in agreement with the findings of Verkleij et al. [8] and Miner and Prestegard [30]. At higher pH the results are more difficult to interpret unequivocally. Verkleij et al. [8] observed a stacked lamellar organization of dioleoylphosphatidate (Ca^{2+}) at pH 8.5. At this pH which is above the second pK_a of dioleoylphosphatidate (pK_a = 8 [7]) dioleoylphosphatidate probably binds calcium in a 1 : 1 stoichiometry, which complex possibly shows a different phase behaviour. On the other hand, Farren et al. concluded from NMR measurements (at pH 8.0) and from freeze-fracture electron microscopy (at pH 7.0), that around the physiological pH Ca^{2+}-dioleoylphosphatidate may be

organized in structures which are intermediate between a lamellar and a hexagonal H_{II} phase [9]. This phase is visualized in electron micrographs as short tube-like structures oriented in a random fashion in the fracture face [9]. Applying X-ray diffraction techniques described by Killian and De Kruijff [31] to a Ca^{2+}-dioleoylphosphatidate sample (the same as was used for ^{31}P-NMR (Fig. 6)) we found a single broad featureless scattering profile (data not shown) with a maximum at $d = 54$ Å. This suggests that dioleoylphosphatidate at pH 7.4 in the presence of calcium (4.5 mM) is organized neither in a hexagonal H_{II} phase nor in an ordered multilamellar phase.

Molecular properties of the sodium salt and the calcium salt of dioleoylphosphatidate

From our DSC results it can be concluded that at pH 7.4 and at room temperature the sodium salt of dioleoylphosphatidate is in the liquid-crystalline phase. Addition of calcium to dioleoylphosphatidate dispersions results in abolition of the gel to liquid-crystalline phase transition in the temperature range of $-35°C$ up to $100°C$, which is in contrast with the results for DOPC and DOPG.

The temperature dependency of the ^2H-NMR spectra of 1,2-[11,11-$^2H_2$]DOPC and the sodium and calcium salt of 1,2-[11,11-$^2H_2$]dioleoylphosphatidate (Figs. 4 and 5) is roughly in agreement with the DSC results (Fig. 1). The increase in $\Delta\nu_q$ of the doublet for deuterated dioleoylphosphatidate (Na^+) upon lowering the temperature to just above the T_c, is a consequence of reduced motion in the system [32]. The dramatic change in lineshape (Fig. 4) and drop in intensity (Fig. 5) of the ^2H-NMR spectra of dioleoylphosphatidate (Na^+) and DOPC at lower temperatures, is typical for a liquid-crystalline to gel phase transition [32–36]. The observed ^2H-NMR lineshapes below T_c originate from non-zero asymmetry parameters arising from a limited mobility of the acyl chains in the gel phase [32]. The ^2H-NMR spectra of the calcium salt of deuterated dioleoylphosphatidate does not show this temperature dependency (Figs. 4 and 5). With decreasing temperature in the range of $-10°C$ to $-30°C$ the spectrum broadens and the integrated echo intensity only gradually decreases, lacking the drop as is found for dioleoylphosphatidate (Na^+) and DOPC (Fig. 5). Also in the range from $25°C$ to $65°C$ neither a steep change in echo intensity nor in lineshape was found (data not shown). The gradual decrease in echo intensity lowering the temperature from $-10°C$ to $-30°C$ suggests that Ca^{2+}-dioleoylphosphatidate might undergo a non-corporative gel to liquid-crystalline phase transition over a broad temperature range which is in agreement with the absence of a phase transition peak in the DSC thermogram. Then at $25°C$ Ca^{2+}-dioleoylphosphatidate is in a liquid-crystalline state, which is confirmed also by the almost complete preservation of ^2H-NMR echo intensity of dioleoylphosphatidate upon calcium addition at this temperature. The above behaviour of Ca^{2+}-dioleoylphosphatidate contrasts with literature data on the temperature dependence of ^2H-NMR intensities of Ca^{2+}-POPG, labeled at the 9,10-position of the oleic acid chain, where a dramatic change in echo integral was found at $48°C$ [33]. DSC data confirmed that in this case a gel to liquid-crystalline phase transition occurred at that temperature [33].

Upon addition of increasing amounts of calcium a broad isotropic signal appears in the ^2H-NMR spectrum of 1,2-[11,11-$^2H_2$]dioleoylphosphatidate, while the original axially symmetric powder pattern gradually disappears (Fig. 3). It is plausible to suggest that both signals originate from two different phosphatidate pools, one in a lamellar organization (sodium salt) and one in a different organization (calcium salt). The typical broad isotropic ^2H-NMR signal of this latter organization (see Fig. 3; 4.5 mM Ca^{2+}) may in principle originate from:

(i) A calcium-induced change from an axially symmetric to an axially asymmetric electric field gradient tensor. However, in view of the liquid-crystalline state of the acyl chains in Ca^{2+}-dioleoylphosphatidate in which restricted motion is doubtful, this explanation is highly unlikely.

(ii) The formation of small structures with a tight radius of curvature in which lateral diffusion of the lipids or particle tumbling further averages the quadrupolar interaction. These structures could be lamellar (i.e. small vesicles) or non-lamellar. The calcium-induced formation of small vesicles seems implausible, since calcium acts as fusogen for phosphatidate-containing vesicles [2,37,38]. Formation of non-bilayer structures allowing iso-

tropic motional averaging of the residual quadrupolar splitting by lateral diffusion is a possible explanation for the broad isotropic ^{2}H-NMR signal. However, the Ca^{2+}-dioleoylphosphatidate complex in excess of aqueous buffer gives rise to a broad ^{31}P-NMR spectrum which only can be observed with ^{31}P cross-polarization techniques (Fig. 4). This spectrum shows a width and lineshape similar to that of the dry powder of Ca^{2+}-glycerophosphate, thus demonstrating that in the calcium salt of dioleoylphosphatidate the phosphate region is fully immobilized. This observation rules out the possibility that lateral diffusion in this system would average the chemical shift anisotropy or the nuclear quadrupolar interaction.

(iii) The formation of structures in which the order of the acyl chains is decreased as a consequence of a disordered configuration of the lipid molecules. We favour this latter possibility as the most plausible explanation of the observed broad isotropic lineshape of the ^{2}H-NMR spectrum of the calcium salt of 1,2-[11,11-$^2H_2$]dioleoylphosphatidate.

Concluding, the following model for the interaction of calcium with dioleoylphosphatidate may explain our observations. Calcium binds to two dioleoylphosphatidate molecules [1] and causes complete immobilization of the polar headgroup, most probably accompanied by dehydration [2,3,25,28], hence the volume which the polar region is occupying is reduced [2,7,38]. The unbalanced proportion between the small dehydrated polar region and the more bulky liquid oleate chains may give the molecule a conical shape. Consequently, it may preferentially organize in highly curved structures in which the polar headgroup is pointing inwards. This may result in the formation of invaginations in the membrane or even inverted structures of dehydrated phosphatidate enclosing calcium, which may explain the electron microscopy observations of Farren et al. [9].

To show the uniqueness of the structural behaviour of dioleoylphosphatidate (Ca^{2+}) a comparison is necessary with the structures of the calcium salts of related acidic phospholipids. From the results in this paper it is clear that at 25°C the physical state and the organization of DOPG are hardly influenced by calcium. The same was observed for (soy bean) PI [39]. For DEPA, DMPA and DPPA, all having a similar polar headgroup as dioleoylphosphatidate, and for DOPS, with the same acyl chains, both headgroup and acyl chain are immobilized upon addition of calcium and the lipid molecules adopt a cochleate organization [11,22,23,40]. Bovine heart cardiolipin, containing a high amount of linoleic acid, remains in a fluid state in the presence of calcium and is organized in a hexagonal H_{II} structure, in which the polar region is highly mobile [28,41]. Apparently, both the nature of the polar headgroup as well as that of the acyl chains is responsible for the broad range of physical states of the negatively charged lipid-Ca^{2+} complexes.

The dioleoylphosphatidate / dioleoylphosphatidylcholine mixed system

In the absence of calcium the enthalpy of the phase transition of the dioleoylphosphatidate/DOPC equimolar mixture is much lower than theoretically expected. Furthermore, the $\Delta\nu_q$ of the ^{2}H-NMR signal of deuterated DOPC is increased upon mixing with dioleoylphosphatidate, while a decrease would be expected. Both observations indicate a different packing of DOPC in the mixed system suggesting a good miscibility of DOPC and dioleoylphosphatidate.

The structural behaviour in the presence of calcium of dioleoylphosphatidate mixed in an equimolar ratio with DOPC shows similarities with the behaviour of pure dioleoylphosphatidate (Ca^{2+}), though higher calcium concentrations are needed to reach the same effect. Below 2 mM of free calcium a fraction of the deuterated dioleoylphosphatidate is already giving rise to an isotropic ^{2}H-NMR signal. From the DSC data, however, it can be concluded that there is no extended phase separation under these conditions, but the decrease in ΔH indicates a withdrawal of a fraction of the lipids (probably Ca^{2+}-dioleoylphosphatidate) from the phase transition. At free calcium concentrations higher than about 2 mM there are most probably two coexisting phases, a phase containing mainly DOPC in a lamellar organization, giving rise to the sharp transition peak, and a dioleoylphosphatidate-rich phase with the same kind of organization as the pure dioleoylphosphatidate calcium salt. The low temperature shoulder

in the DSC scans may originate from a fraction of the DOPC which is influenced by the presence of Ca^{2+}-dioleoylphosphatidate supporting the idea that calcium has a fluidizing effect. Next the contribution of the axially symmetric powder pattern in the ^{2}H-NMR spectra of deuterated dioleoylphosphatidate is diminished further. At higher calcium concentrations DOPC apparently gets progressively more involved in the calcium-phosphatidate rich phase indicated by the collapse of the sharp transition peak and the further reduction of the total transition enthalpy, implying a remixing of the lipids. At these calcium concentrations the ^{2}H-NMR signal of deuterated DOPC shows similarities with that of dioleoylphosphatidate (Ca^{2+}), suggesting the same disordering in the oleate chains of the phosphatidylcholine. Moreover, the loss of ^{31}P-NMR signal indicates that the polar headgroup of a proportion of the lipid molecules, probably dioleoylphosphatidate (Ca^{2+}), is immobilized and, presumably, dehydrated.

Graham et al. [11] studied the calcium induced phase separation in phosphatidic acid/phosphatidylcholine equimolar systems using fluorescence quenching techniques. Their results for dioleoylphosphatidate/DOPC, showing a very rapid phase separation between the two lipids at 10 mM of calcium, are in good agreement with our findings. Using electron microscopy Hartmann et al. [10] visualized a calcium-induced phase separation in dioleoylphosphatidate/DOPC (50 : 50, mol%) vesicles, but, in contrast with our interpretation here, they concluded that formation of a rigid dioleoylphosphatidate (Ca^{2+}) phase had occurred.

Our DSC results for the dioleoylphosphatidate/DOPC (20 : 80, mol%) mixture also suggest a phase separation or at least the formation of a phase enriched in DOPC. From the ^{2}H-NMR it is clear that a fraction of the dioleoylphosphatidate molecules is affected by the presence of calcium, and, according to our interpretation, have the same kind of structure as the calcium salt of pure dioleoylphosphatidate. Apparently even at low millimolar concentrations of calcium a fraction of the dioleoylphosphatidate molecules to which calcium is bound is able to adopt its own preferred organization. It should be noticed, however, that the observed effects of calcium on this mixture are minor compared to the effects on the equimolar mixture of the two lipids. While with DSC some effect is measurable, ^{31}P-NMR does not result in any observable changes upon the addition of calcium to dioleoylphosphatidate/DOPC (20 : 80, mol%).

The phosphatidate calcium shuttle in dioleoylphosphatidate / dioleoylphosphatidylcholine (20 : 80, mol%) model membranes

On the ground of recent publications it can be stated that a general consensus has been reached on the ability of phosphatidate to translocate calcium selectively across model membranes [1–5,19,42]. In an earlier study [2] we proposed a dehydrated complex of calcium and phosphatidate as the translocating shuttle for dioleoylphosphatidate/DOPC (20 : 80, mol%) membranes. In this we support the view of Chauhan and Brockerhoff [3,19], although we did not exclude that more than two phosphatidate molecules and, consequently, more than one calcium ion are participating in the translocation complex [2]. The results and interpretations of the present study give additional support for the following hypothesis on the structural aspects of phosphatidate mediated transmembrane traversal of calcium.

When calcium binds to phosphatidate in a dioleoylphosphatidate/DOPC (20 : 80, mol%) membrane, local clusters may be formed of two or more phosphatidate molecules. The negative charge of phosphatidate is neutralized by calcium and the polar headgroup is dehydrated thus rendering the complex more hydrophobic than its individual components. The shape of the complex with a polar region which is decreased in volume may give rise to local increase in curvature of the membrane surface resulting in the formation of inverted structures of Ca^{2+}-dioleoylphosphatidate. These structures with a calcium-containing anhydrous core and an apolar exterior may dissolve in the hydrophobic part of the bilayer and thus pass the membrane. This proposed inverted conformation is essentially different from the inverted micellar structure, which is thought to play a role in cardiolipin-mediated transmembrane traversal [43,44]. These inverted micelles are suggested to enclose an aqueous compartment similar to that found in the related hexagonal H_{II} phase and can

be visualized by freeze fracture electron microscopy as particles in the interior of the membrane [44]. Because of the anhydrous core the Ca^{2+}-dioleoylphosphatidate structures should be much smaller than the inverted micellar structures.

The possibility that other phospholipids (PG, PE and PC) are participating in the calcium phosphatidate complex, as was postulated by Reusch [4], is not supported by our findings. DOPC only takes part in the dioleoylphosphatidate (Ca^{2+}) phase at high calcium concentrations. Furthermore, we have previously found that addition of calcium to large unilamellar dioleoylphosphatidate/DOPC (20 : 80, mol%) vesicles did not result in an increased transbilayer movement of DOPC [2].

Conclusion

Upon addition of calcium the phosphate region of dioleoylphosphatidate is fully immobilized whereas the acyl chains remain liquid crystalline but become more disordered. The pure system of the calcium salt of dioleoylphosphatidate is neither organized in a hexagonal H_{II} phase nor in ordered lamellar structures. In mixtures of DOPC and dioleoylphosphatidate calcium apparently induced the formation of a phase rich in Ca^{2+}-dioleoylphosphatidate in which the complex behaves similarly to pure Ca^{2+}-dioleoylphosphatidate. From the data it can be hypothesized that phosphatidate-mediate selective calcium translocation across DOPC model membranes occurs via phosphatidate calcium shuttles which are inverted anhydrous structures of phosphatidate enclosing calcium ions.

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